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Effect of Calmodulin on Ginseng Saponin-Induced Ca^{2+}-Activated Cl^- Channel Activation in *Xenopus laevis* Oocytes

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We previously demonstrated the ability of ginseng saponins (active ingredients of *Panax* ginseng) to enhance Ca^{2+}-activated Cl^- current. The mechanism for this ginseng saponin-induced enhancement was proposed to be the release of Ca^{2+} from IP_3-sensitive intracellular stores through the activation of PTX-insensitive $G\alpha_{q/11}$ proteins and PLC pathway. Recent studies have shown that calmodulin (CaM) regulates IP_3 receptor-mediated Ca^{2+} release in both Ca^{2+}-dependent and -independent manner. In the present study, we have investigated the effects of CaM on ginseng saponin-induced Ca^{2+}-activated Cl^- current responses in *Xenopus* oocytes. Intraoocyte injection of CaM inhibited ginseng saponin-induced Ca^{2+}-activated Cl^- current enhancement, whereas co-injection of calmidazolium, a CaM antagonist, with CaM blocked CaM action. The inhibitory effect of CaM on ginseng saponin-induced Ca^{2+}-activated Cl^- current enhancement was dose- and time-dependent, with an IC_{50} of 14.9 ± 3.5 μM. The inhibitory effect of CaM on saponin's activity was maximal after 6 h of intraoocyte injection of CaM, and after 48 h the activity of saponin recovered to control level. The half-recovery time was calculated to be 16.7 ± 4.3 h. Intraoocyte injection of CaM inhibited Ca^{2+}-induced Ca^{2+}-activated Cl^- current enhancement and also attenuated IP_3-induced Ca^{2+}-activated Cl^- current enhancement. Ca^{2+}/CaM kinase II inhibitor did not inhibit CaM-caused attenuation of ginseng saponin-induced Ca^{2+}-activated Cl^- current enhancement. These results suggest that CaM regulates ginseng saponin effect on Ca^{2+}-activated Cl^- current enhancement *via* Ca^{2+}-independent manner.

Key words: *Panax* ginseng, Calmodulin, Ca^{2+}-Activated Cl^- channel, *Xenopus* oocytes

INTRODUCTION

Ginseng, the root of *Panax ginseng* C.A. Meyer, is a well-known folk medicine as a tonic and restorative agent. Ginsenosides are main molecular ingredients of ginseng, and are responsible for ginseng's activity. Ginsenoside's structure is comprised of aglycone and carbohydrates portions. Aglycone is the main backbone of ginsenoside with a hydrophobic four-ring steroid-like structure. The carbohydrates linked to aglycone consist of monomer, dimer, or tetramer. Thus, they are amphiphilic with hydrophilic carbohydrates and hydrophobic backbone structure (Nah, 1997).

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Intracellular Ca^{2+} is a main molecule for signal transduction pathway in a variety of cells. Increase in Ca^{2+} level in cells regulates secretion, cell division, growth and differentiation, muscle contraction, and receptor internalization (Berridge *et al.*, 1998). Intracellular free Ca^{2+} can be increased either by Ca^{2+} influx from extracellular fluid or through the release of stored Ca^{2+} from intracellular compartment, called endoplasmic reticulum (ER) (Parekh and Penner, 1997). In non-excitable cells, an increase in cytoplasmic free Ca^{2+} is mediated *via* receptor stimulation such as G protein coupled receptors, which are coupled to activation of phospholipase C (PLC) pathway and production of IP_3. The produced IP_3 triggers an increase in the levels of cytosolic free Ca^{2+} from IP_3-sensitive store ERs, resulting in activation of Ca^{2+}-dependent intracellular processes (Kasri *et al.*, 2002; Taylor and Laude, 2002). For example, stimulation of oocyte muscarinic receptors by

ACh leads to intracellular Ca^{2+} mobilization and activation of Ca^{2+}-activated Cl^- channels, resulting in depletion of calcium stores in *Xenopus laevis* oocytes (Dascal *et al.*, 1984; Berridge and Irvine, 1989; Lechleiter and Clapham, 1992).

Similarly, Choi *et al.* (2001a, b) and Jeong *et al.* (2004) also demonstrated that, in *Xenopus* oocytes, treatment of ginseng saponin initiates the activation of $G\alpha_{q/11}$-phospholipase C (PLC)-β3 pathway coupled to IP_3-mediated intracellular Ca^{2+} release, resulting in activation of Ca^{2+}-actvated Cl^- channels.

It is known that calmodulin (CaM) is a Ca^{2+}-binding protein and a Ca^{2+}-sensor. CaM is abundantly present in many eukaryotic cell types (Gnegy, 1993). The main functions of CaM are to function as a Ca^{2+}-dependent regulator of various enzymes like kinases or phosphatases, in voltage-dependent Ca^{2+} channels and Ca^{2+} pumps, and in cytoskeletal elements (Liu *et al.*, 1994). Recent reports have shown that CaM binds to IP_3 receptors in ER as both Ca^{2+}-CaM and apo-CaM. CaM binding sites in IP_3 receptors are basic amphipathic α-helical structure bearing homology to many other Ca^{2+}-CaM-binding sites (Yamada *et al.*, 1995; Cardy *et al.*, 1998; Sienaert *et al.*, 2002), CaM, not only acts as an endogenous inhibitor for IP_3 receptor, but also inhibits IP_3 binding to its IP_3 receptor in both Ca^{2+}-dependent and -independent manner (Yamada *et al.*, 1995; Cardy *et al.*, 1998; Sienaert *et al.*, 2002).

Ginseng saponin has also been reported to induce IP_3-mediated Ca^{2+} release from ERs for the activation of Ca^{2+}-activated Cl^- channel in *Xenopus* oocytes (Choi *et al.*, 2001a,b). But there are no direct evidences that CaM could also modulate ginseng saponin-mediated Ca^{2+}-activated Cl^- chancel activation. Hence, in the present study, we have investigated that how CaM regulates ginseng saponin-induced Ca^{2+}-activated Cl^- current enhancement in *Xenopus* oocytes, which are convenient for intracellular injection of putative second messengers and can substantially facilitate investigations of intermediate steps in signaling pathways due to their large size and easy handling. We have reported that intraoocyte injection of CaM inhibited ginseng saponin-induced Ca^{2+}-actvated Cl^- current enhancement in a dose- and time-dependent manner. The inhibitory effect of CaM was not blocked by intraoocyte injection of Ca^{2+} or IP_3. These results indicate that in *Xenopus* oocytes, CaM regulates ginseng saponin activity on Ca^{2+}-activated Cl^- channel activation *via* Ca^{2+}- and IP_3-insensitive manner.

MATERIALS AND METHODS

Drugs

Fig. 1A shows the structures of the eight representative ginseng saponins. The ginseng total saponins (GTS) were a kind gift from Korea Ginseng Cooperation (Taejon, Korea). GTS contained Rb_1 (17.1%), Rb_2 (9.07%), Rc (9.65%), Rd (8.26%), Re (9%), Rf (3%), Rg_1 (6.4%), Rg_2 (4.2%), Rg_3 (3.8%), Ro (3.8%), Ra (2.91%) and other minor ginsenosides. GTS was diluted with bath medium, ND96 before use. Calmodulin (CaM), calmidazolium hydrochloride, and CaMKII inhibitor peptide (281-309) were purchased from Calbiochem. The drugs used in this study were either bath-applied or injected into oocytes with a Nanoject Automatic Oocyte Injector (Drummond Scientific, PA, USA). The injection pipette was pulled from glass capillary tubing, and its tip was broken to an outer diameter of about 20 μm, and about 23-50 mL of CaM was injected into oocytes, depending on the concentration levels.

Oocyte preparation

Xenopus laevis frogs were obtained from Xenopus I (Ann Arbor, MI, USA). Before being operated for oocyte extraction, the frogs were kept in a temperature-controlled aquarium (18 ± 1°C) with a 12:12 h light-dark cycle, and food was given every two days. Oocytes were extracted under deep anesthesia, induced by immersing frogs in an aerated solution of 0.15% 3-amino benzoic acid ethyl ester. Following oocyte extraction, frogs were sacrificed by overdosing the anesthetic level. The extracted oocytes were separated by treatment with collagenase and agitation for 2 h in a Ca^{2+}-free medium containing 82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, 2.5 mM sodium pyruvate, 100 units/mL penicillin and 100 μg/mL streptomycin. Stage V-VI oocytes were collected and stored in ND96 (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 5 mM HEPES, pH 7.5) supplemented with 0.5 mM theophylline and 50 μg/μL gentamicin. The oocyte containing solution was maintained at 18°C with continuous gentle shaking and was changed on a daily basis.

In vitro synthesis of cRNA

Recombinant plasmids containing cDNA inserts of m1 mAChR were linearized by digestion with appropriate restriction enzymes. The cRNAs from linearized templates were obtained with an *in vitro* transcription kit (mMessage mMachine; Ambion, Austin, TX, USA) using T7 RNA polymerase. The RNA was dissolved in RNase-free water at 1 μg/μL, divided into aliquots and stored at -70°C until used.

Electrophysiological recording

A custom-made Plexiglas net chamber was used for two-electrode voltage-clamp recordings. The chamber was constructed by milling two concentric wells into the chamber bottom (diameter/height: upper well: 8/3 mm, lower well: 6/5 mm) and gluing plastic meshes (~0.4-mm grid diameter) onto the bottom of the upper well. The perfusion

inlet (~1-mm diameter) was formed through the wall of the lower well, and the suction tube was placed on the edge of the upper well. Oocyte was placed on the net that separates the upper from the lower wells. The grids of the net served as dimples that kept the oocyte in place during electrophysiological recordings. Oocytes were impaled with two microelectrodes filled with 3 M KCl (0.2-0.7 MΩ). The electrophysiological experiments were done at room temperature with Oocyte Clamp (OC-725C, Warner Instrument, CT) and stimulation and data acquisition were controlled by pClamp 8 (Axon Instruments) (Lee *et al.*, 2004).

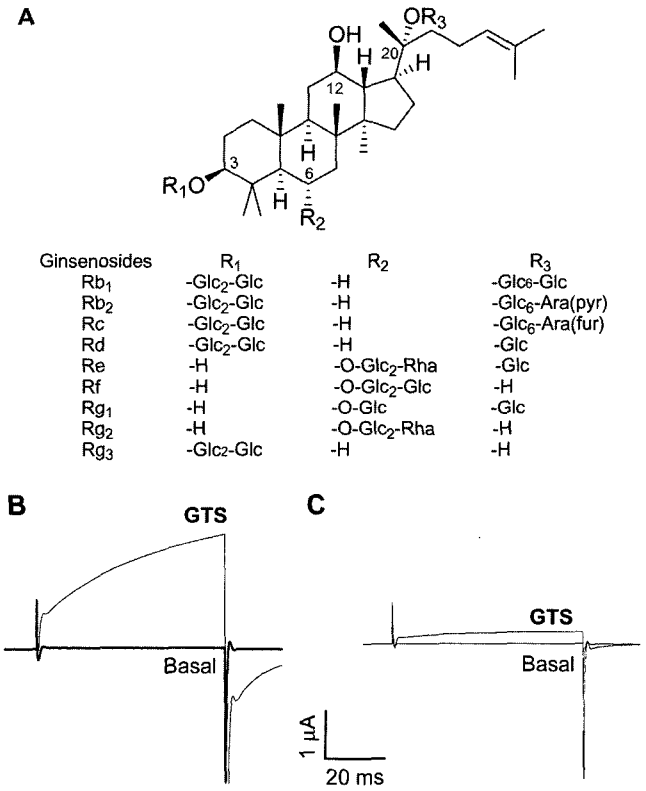
Data analysis

To obtain the concentration-response curve in the presence of CaM, the observed peak amplitudes were normalized, plotted and then fitted to the Hill equation as given below using Origin software (Northampton, MA). $y/y_{max} = [A]^n/([A]^n + [EC_{50}]^n)$, where y is % inhibition at given concentration of CaM, y_{max} is the maximal peak current, IC_{50} is the concentration of CaM producing half-maximum effect of the control response to ginsenosides, [A] is the concentration of CaM, and n is the interaction coefficient. All values are presented as mean ± S.E.M. The differences between means of control and treatment data were analyzed by unpaired *t*-test. $P < 0.05$ was considered significant.

RESULTS

Effect of intraoocyte-injected CaM on ginseng saponin-induced Cl^- current responses in *Xenopus* oocytes

In previous report, we have demonstrated that ginseng saponins induced an activation of Ca^{2+}-actvated Cl^- channels *via* $G\alpha_{q/11}$-phospholipase C (PLC)-β3 pathway coupled to IP_3-mediated intracellular Ca^{2+} release in a dose-dependent manner (Choi *et al.*, 2001a; Jeong *et al.*, 2004), and in the present study it was observed that ginseng saponins also increased Ca^{2+}-actvated Cl^- current in a dose-dependent manner (data not shown). We further examined the changes in ginseng saponin-induced Ca^{2+}-actvated Cl^- current responses after intraoocyte injection of calmodulin (CaM). As shown in Fig. 1B, treatment of ginseng saponins (50 μg/mL) to H_2O-injected control oocytes induced a large Ca^{2+}-activated Cl^- current enhancement, whereas intraoocyte injection of CaM (40 μM, final) for 6 h almost blocked ginseng saponin-induced Ca^{2+}-activated Cl^- current enhancement (Fig. 1C). For further characterizations of the effect of CaM on ginseng saponin action, we performed current-voltage relationship experiments. As shown in previous reports, in the present case also it was observed that treatment of ginseng saponins



Ginsenosides	R_1	R_2	R_3
Rb_1	-Glc_2-Glc	-H	-Glc_6-Glc
Rb_2	-Glc_2-Glc	-H	-Glc_6-Ara(pyr)
Rc	-Glc_2-Glc	-H	-Glc_6-Ara(fur)
Rd	-Glc_2-Glc	-H	-Glc
Re	-H	-O-Glc_2-Rha	-Glc
Rf	-H	-O-Glc_2-Glc	-H
Rg_1	-H	-O-Glc	-Glc
Rg_2	-H	-O-Glc_2-Rha	-H
Rg_3	-Glc2-Glc	-H	-H

Fig. 1. Structure of the nine representative ginsenosides and the effect of calmodulin (CaM) on ginsneosides (GTS)-induced Ca^{2+}-activated Cl^- current enhancement. They differ at three side chains attached to the common steroid ring (A). Abbreviations for carbohydrates are as follows: Glc, glucopyranoside; Ara(pyr), arabinopyranoside; Rha, rhamnopyranoside, Superscripts indicate the carbon in the glucose ring that links the two carbohydrates. Treatment of ginsenosides (GTS) (50 μg/mL) induces a large Ca^{2+}-activated Cl^- channel activation in H_2O-injected control oocytes (B), whereas intraoocyte injection of CaM (40 μM, final) for 6 h blocks the GTS-induced Ca^{2+}-activated Cl^- current enhancement (C). The current responses were evoked every 5 s by a voltage step from -80 mV to +60 at the holding potential (V_h) of -80 mV. Tracings are representative of six separate oocytes from three different frogs.

increased Ca^{2+}-activated Cl^- currents in a voltage-dependent manner. The reversal potential was close to –20 mV, indicating that ginseng saponins activate endogenous Ca^{2+}-activated Cl^- channels in *Xenopus* oocytes (Fig. 2A) (Choi *et al.*, 2001a,b; Lee *at al.*, 2004). However, preintraoocyte injection of CaM for 6 h attenuated ginseng saponin-induced Ca^{2+}-activated Cl^- current enhancement. We also tested the effect of CaM in oocytes expressing m1 muscarinic ACh receptor. In oocytes expressing m1 muscarinic ACh receptor, treatment of ACh (100 μM) also enhanced Ca^{2+}-activated Cl^- current, as shown in previous report (Lee *et al.*, 2004) but preintraoocyte injection of CaM for 6 h also blocked the effect of ACh on Ca^{2+}-activated Cl^- current enhancement (Fig. 2B). However, co-injection of CaM with calmidazolium, a CaM antagonist, blocked CaM effect on ginseng saponin-induced Cl^-

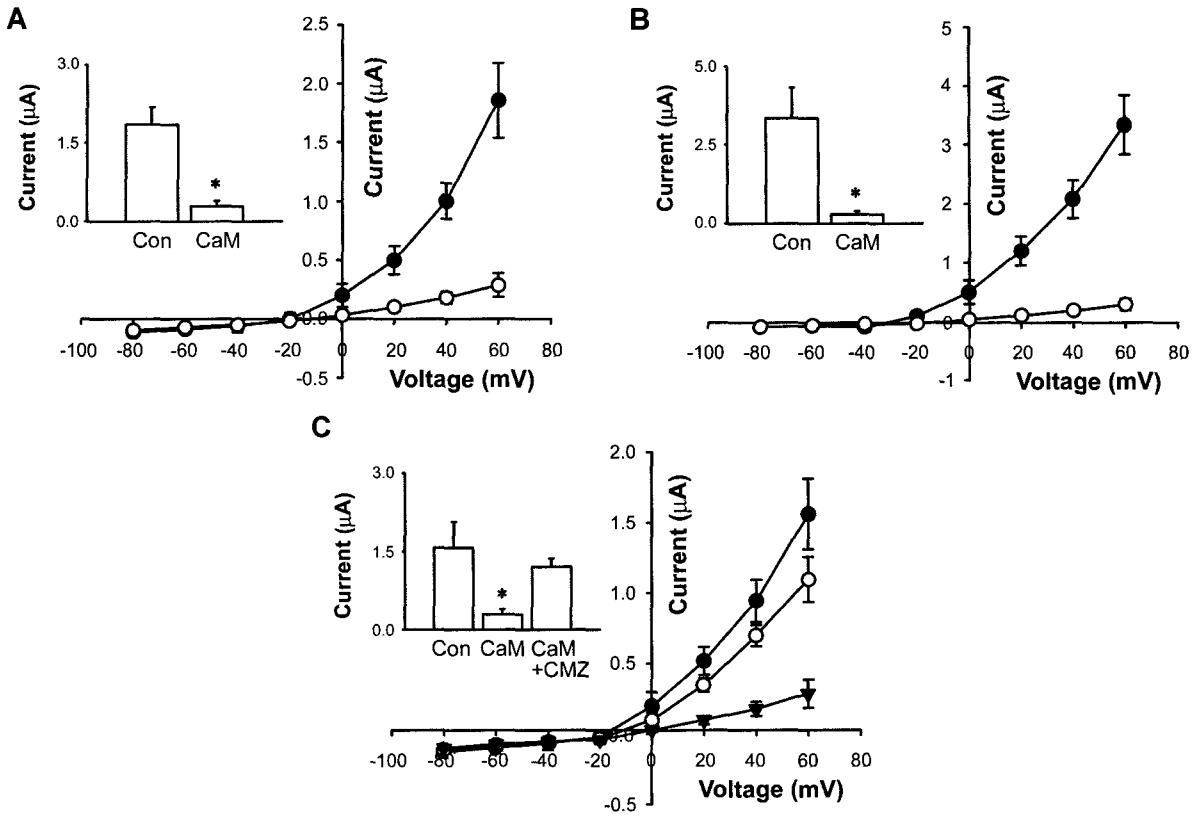


Fig. 2. Effects of intraoocyte injected CaM on GTS- or acetylcholine (ACh)-induced Ca^{2+}-activated Cl^- current enhancement (A) Current-voltage (I-V) curves for the data between H_2O-injected control (●) and CaM (40 μM, final) (○)-injected oocytes in the presence of GTS (50 μg/mL). The current responses were evoked by voltage steps (20-mV increment from -80 mV to +60 mV) at the holding potential (V_h) of -80 mV. *Inset,* the peak mean amplitudes of the outward currents recorded in the presence of GTS after injection of H_2O (Con) or CaM (mean ± S.E.M; n=15 oocytes each). The data denoted with an *asterisk* was significantly different from H_2O-injected control oocytes (*$p < 0.001$). (B) Oocytes were injected with cRNAs (20 ng/oocyte) coding m1 muscarinic ACh receptor for two days. Current-voltage (I-V) curves for the data between H_2O-injected control (●) and CaM (40 μM, final) (○)-injected oocytes in the presence of 100 μM ACh. The current responses were evoked by voltage steps (20-mV increment from -80 mV to +60 mV) at the holding potential (V_h) of -80 mV. *Inset,* the peak mean amplitudes of the outward currents recorded in the presence of GTS after injection of H_2O or CaM (mean ± S.E.M; n=15 oocytes each). The data denoted with an *asterisk* was significantly different from H_2O-injected control oocytes (*$p < 0.01$). (C) Current-voltage (I-V) curves for the data between H_2O (Con, ●)-, CaM (▼)-, or CaM + calmidazolium (100 μM, final) (○)-coinjected oocytes in the presence of GTS (50 μg/mL). The current responses were evoked by voltage steps (20-mV increment from -80 mV to +60 mV) at the holding potential (V_h) of -80 mV. *Inset,* the peak mean amplitudes of the outward currents recorded in the presence of GTS after injection of H_2O, CaM, or calmidazolium + CaM (mean ± S.E.M; n=15 oocytes each). The data denoted with an *asterisk* was significantly different from CaM-injected oocytes (*$p < 0.01$).

current response (Fig. 2C) (Adkins *et al.*, 2000).

Concentration- and time-dependent effect of intraoocyte-injected CaM on ginseng saponin-induced Ca^{2+}-activated Cl^- current responses in *Xenopus oocytes*

Fig. 3A shows concentration-dependent response curve for CaM-induced inhibition on ginseng saponin-induced Ca^{2+}-activated Cl^- current enhancement. Intraoocyte injection of CaM inhibited ginsenoside-induced Ca^{2+}-activated Cl^- current responses in a dose-dependent manner, with an IC_{50} of 14.9±3.5 μM. This value was very similar to the value determined for experiments demonstrating inhibition of IP_3-evoked Ca^{2+} mobilization by CaM in SH-SY5Y cells (Adkins *et al.*, 2000). Fig. 3B also shows the time dependent inhibitory effect of CaM on ginseng saponin-induced Ca^{2+}-activated Cl^- channel enhancement. The inhibitory effect of CaM was maximal after 6 h of intraoocyte injection and the effect was persistent for at least 12 h. The ginsenoside-induced Ca^{2+}-activated Cl^- current enhancement was recovered to control level after 48 h of intraoocyte injection of CaM. The half recovery time was 16.7 ± 4.3 h.

Effect of CaM on Ca^{2+} or IP_3-induced Ca^{2+}-activated Cl^- channel activation

The previous reports have shown that direct intraoocyte injection of Ca^{2+} or agents increasing intraoocyte free Ca^{2+}

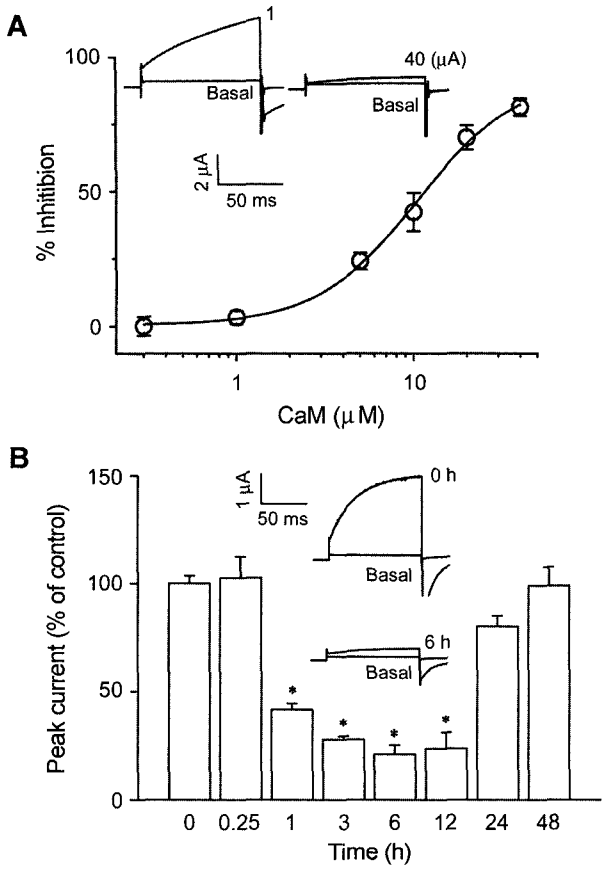


Fig. 3. Concentration- and time-dependent effect of CaM on GTS-induced Ca^{2+}-activated Cl^- current enhancement. (A) Oocytes were injected with different concentrations of CaM and incubated for 6 h in ND96. Other experimental procedures were same as described in Fig. 1. *Inset,* peak outward currents (mean ± S.E.M; n=14-16 each) recorded in oocytes injected with H_2O or 40 μM CaM in the presence of GTS (50 μg/mL). The curve described by the solid line was fit by the Hill equation as described in Method sections. (B) The oocytes were injected with H_2O (Con) or 40 μM CaM and incubated for the indicated time in ND96. The peak amplitudes of the outward currents were recorded as per the method described in Fig. 1 (mean±S.E.M; n=13-15 oocytes each). Those denoted with asterisk were significantly different from the others (*$p < 0.001$).

concentration such as IP_3 or Ca^{2+} ionophore induced activation of Ca^{2+}-activated Cl^- channel (Parekh, 1995; Hartzell, 1996; Kuruma and Hartzell, 1999). Since CaM is a Ca^{2+} binding protein and also an IP_3 receptor regulator, we investigated whether intraoocyte injected CaM might also affect Ca^{2+}-activated Cl^- current enhancement induced by intraoocyte injection of Ca^{2+} or IP_3. As shown in Fig. 4A (*left panel*), intraoocyte injection of CaM attenuated ginseng saponin response on Ca^{2+}-activated Cl^- current, when compared to H_2O-injected control oocytes. Intraoocyte injection of Ca^{2+} (5 mM, final) to the oocytes that have been injected with H_2O for 6 h, induced a large Ca^{2+}-activated Cl^- current enhancement, whereas intraoocyte

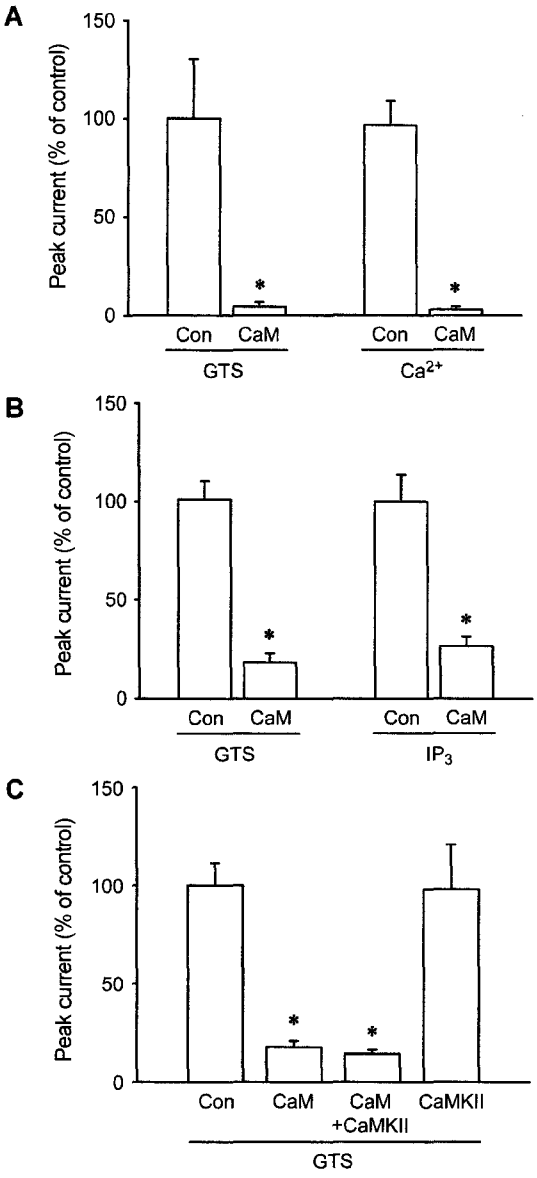


Fig. 4. Effect of Ca^{2+}, IP_3, or Ca^{2+}/CaM kinase II (CaMKII) inhibitor on CaM-caused attenuation of ginsenoside-induced Cl^- current enhancement (A) Histograms of peak outward current after bathing application of GTS (50 μg/mL) to oocytes that were injected with H_2O (Con), CaM alone, CaM + Ca^{2+} or Ca^{2+} alone. In groups of CaM + Ca^{2+}, intraoocyte injection of Ca^{2+} (5 mM) was performed 6 h after CaM injection. Other experimental procedures were same as described in Fig. 1(B) Histograms of peak outward current after bathing application of GTS (50 μg/ml) to oocytes that were injected with H_2O (Con), CaM alone, CaM + IP_3 or IP_3 alone. In groups of CaM + IP_3, intraoocyte injection of IP_3 (100 μM) was performed 6 h after CaM injection. Other experimental procedures were same as described in Fig. 1. (C) Histograms of peak outward current after bathing application of GTS (50 μg/mL) to oocytes that were injected with H_2O (Con), CaM alone, CaM + CaMKII inhibitor or CaMKII inhibitor alone. In experiments using CaMKII inhibitor + CaM, CaMKII inhibitor was co-injected with CaM. Other experimental procedures were same as described in Fig. 1. Other experimental procedures were same as described in Fig. 1. Those denoted with asterisk were significantly different from the others (*$p < 0.001$).

injection of Ca^{2+} (5 mM, final) to the same oocytes that have been injected with CaM (final 40 μM) for 6 h failed to enhance Ca^{2+}-activated Cl^- current. We found that intraoocyte injection of low concentration of Ca^{2+} (0.1 μM, final) had no effect on CaM action (data not shown). Subsequently, we investigated the effect of intraoocyte injected CaM on IP_3-induced Ca^{2+}-activated Cl^- channel activation. As shown in Fig. 4B, intraoocyte injection of CaM (final 40 μM) for 6 h blocked ginseng saponin-induced Ca^{2+}-activated Cl^- current enhancement (Fig. 4B, *left panel*). Moreover, intraoocyte injected CaM also blocked IP_3 (100 μM, final)-induced Ca^{2+}-activated Cl^- current enhancement, although intraoocyte injection of IP_3 to H_2O-injected control oocytes induced a large Ca^{2+}-activated Cl^- current enhancement (Fig. 4B). Intraoocyte injection of Ca^{2+} (5 mM, final) or IP_3 (100 μM, final) to the same oocytes that have been injected with CaM for 6 h also did not affect CaM-caused attenuation of ginseng saponin-induced Cl^- current response (data not shown). These results indicate that intraoocyte injection of Ca^{2+} or IP_3 induces Ca^{2+}-activated Cl^- channel activation, whereas intraoocyte injected CaM inhibits not only the activity of ginseng saponin, but also affects Ca^{2+}- or IP_3-induced Ca^{2+}-activated Cl^- channel activation.

Effect of CaMKII inhibitor on CaM-caused attenuation of ginseng saponin-induced Cl^- current enhancement

Subsequently, we examined the effect of CaMKII inhibitor on CaM-caused inhibition on ginseng saponin-induced Ca^{2+}-activated Cl^- current responses, since intraoocyte injected CaM might form a complex of Ca^{2+}/CaM with cytosolic free Ca^{2+} and activate CaMKII in oocytes, and the activated CaMKII might phosphorylate/regulate IP_3 receptor functions (Parys *et al.*, 1992). The other possibility was that the activated Ca^{2+}/CaMKII might also phosphorylate Ca^{2+}-activated Cl^- channels that respond to ginseng saponin treatment and affect ginseng saponin-induced Cl^- current enhancement. To test this hypothesis, we also examined the effect of ginseng saponin on Ca^{2+}-activated Cl^- current response after intraoocyte co-injection of Ca^{2+}/CaMKII inhibitor peptide (281-309) (final 100 μM) with CaM (Matifat *et al.*, 1997). As shown in Fig. 4C, intraoocyte injected Ca^{2+}/CaMKII inhibitor peptide (281-309) with CaM for 6 h had no effect on CaM-caused inhibition of ginseng saponin-induced Ca^{2+}-activated Cl^- current enhancement (Fukunaga *et al.*, 1993; Waxham and Aronowski *et al.*, 1990).

DISCUSSION

Activation of G protein-coupled receptors, which are endogenous or heterologously expressed in *Xenopus* oocytes, initiates a signaling cascade that leads to the opening of Ca^{2+}-activated Cl^- channels after release of intracellular free Ca^{2+} from ER through PLC-IP_3 pathway (Dascal *et al.*, 1984; Berridge and Irvine, 1989; Lechleiter and Clapham, 1992). Similarly, we have also shown that, in *Xenopus* oocytes, ginseng saponin interaction with membrane components at the extracellular side enhanced a Ca^{2+}-activated Cl^- current and that this process involved the activation of PTX-insensitive G protein and PLC and the release of Ca^{2+} from IP_3-sensitive intracellular stores (Choi *et al.*, 2001a,b; Jeong *et al.*, 2004). The present study was further performed to explain the role of CaM in ginseng saponon-mediated Ca^{2+}-activated Cl^- current enhancement, since CaM is a regulator of IP_3 receptor-mediated intracellular Ca^{2+} release (Missianen *et al.*, 1999; Adkins *et al.*, 2000). In this study, we have provided three independent evidences about CaM acting as a regulator of ginseng saponin-induced Ca^{2+}-activated Cl^- channel activation. First, we showed that the inhibitory effect of CaM on ginseng saponin-induced Ca^{2+}-activated Cl^- current response was CaM-specific, since CaM action on ginseng saponin effect was blocked by CaM antagonist, calmidazolium (Fig. 2). Second, we showed that intraoocyte injected CaM blocked ginseng saponin-induced Ca^{2+}-activated Cl^- current response in a concentration-dependent manner. Third, the inhibitory effect of CaM on ginseng saponin-induced Ca^{2+}-activated Cl^- current enhancement was persistent but reversed slowly, since ginseng saponin effect on Ca^{2+}-activated Cl^- current enhancement completely recovered 48 h after intraoocyte injection of CaM. Thus, the half-recovery time for ginseng saponin effect on Ca^{2+}-activated Cl^- channel was about 17 h, suggesting that the binding affinity of CaM to IP_3 receptor or other regulatory site(s) might be high.

In addition, we found that the inhibitory effect of CaM on ginseng saponin-induced Ca^{2+}-activated Cl^- current enhancement might be in a Ca^{2+}-independent manner, since intraoocyte injection of Ca^{2+} did not block CaM action (Fig. 4). Recent reports also showed that CaM regulates IP_3 receptor-mediated Ca^{2+} release in both Ca^{2+}-dependent and -independent manner (Yamada *et al.*, 1995; Cardy *et al.*, 1998; Sienaert *et al.*, 2002). Thus, the present study showed a possibility that CaM-induced regulation of ginseng saponin-mediated Ca^{2+}-activated Cl^- current enhancement might be achieved *via* Ca^{2+}-independent manner. We showed that intraoocyte injection of IP_3 did not prevent CaM action, indicating that the inhibitory effect of CaM on ginseng saponin-induced Ca^{2+}-activated Cl^- current enhancement was not interfered by the presence of IP_3, thus suggesting that CaM inhibits IP_3 receptor-mediated Ca^{2+} release by regulating other regulatory domain(s) that are different from that of IP_3 binding sites. In fact, CaM binding domain in IP_3 receptor is different from that of IP_3 binding

sites (Kasri et al., 2002; Taylor and Laude, 2002). We also showed that CaM-mediated inhibition on ginseng saponin-induced Ca^{2+}-activated Cl^- current enhancement was not achieved *via* Ca^{2+}/CaMKII activation, since Ca^{2+}/CaMKII inhibitor peptide (281-309) did not block the action of CaM.

Previous reports have shown that CaM binds to IP_3 receptors in ER and interferes with IP_3 receptor activation (Yamada *et al.*, 1995; Cardy *et al.*, 1998; Sienaert *et al.*, 2002). Thus, one possibility is that intraoocyte injected CaM interrupts the action of IP_3 that are formed after ginseng saponin treatment and blocks Ca^{2+} release from ER. As a result, there is no Ca^{2+}-activated Cl^- current response to ginseng saponin treatment (Figs. 2 and 3). Supporting this notion is the fact that intraoocyte injection of IP_3 to the same oocytes that were previously injected with CaM for 6 h failed to evoke Ca^{2+}-activated Cl^- current to the same extent as that of IP_3 in H_2O-injected control oocytes (Fig. 4B). As supporting evidence, Missiaen *et al.* (1999) and Adkins *et al.* (2000) also showed that CaM inhibits IP_3 receptor-mediated Ca^{2+} release in permeabilized A7r5 and SH-SY5Y cells, respectively. The other possibility is that Ca^{2+} binding property of CaM might also attenuate ginseng saponin-induced Ca^{2+}-activated Cl^- current enhancement. Thus, intraoocyte injected CaM might quench cytosolic free Ca^{2+}s that are released from ER after ginseng saponin treatment. However, this explanation is unlikely, since one would expect that ginseng saponin treatment or intraoocyte injected Ca^{2+} would also enhance Ca^{2+}-activated Cl^- current in oocytes injected with CaM when exogenously excessive Ca^{2+} was administered into oocytes (Fig. 4A). These results suggest further that CaM regulates ginseng saponin-induced Ca^{2+}-activated Cl^- current enhancement in a Ca^{2+}-independent manner. Thus, there might be more than one mechanism in CaM-caused attenuation of ginseng saponin-induced Ca^{2+}-activated Cl^- current enhancement. Further studies are required to clarify the role of CaM in ginseng saponin-induced Ca^{2+}-activated Cl^- current enhancement.

In summary, using a *Xenopus* oocyte model system for explanation of ginseng saponin signaling pathway, we obtained further results on the involvement of CaM in ginseng saponin-induced Ca^{2+}-activated Cl^- channel regulation. CaM may be one of modulators in signaling pathways that underlie *Panax* ginseng action.

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REFERENCES

Adkins, C. E., Morris, S. A., De Smedt, H., Sienaert, I., Torok, K., and Taylor, C. W., Ca^{2+}-calmodulin inhibit Ca^{2+} release mediated by type-1, -2, and -3 inositol trisphosphate receptors. *Biochem. J.*, 345, 357-363 (2000).

Berridge, M. J. and Irvine, R. F., Inositol triphosphates and cell signalling. *Nature*, 341, 197-205 (1989).

Berridge, M. J., Bootman, M. D., and Lipp, P., Calcium - a life and death signal. *Nature* (Lond.), 395, 645-648 (1998).

Cardy, T. J. A. and Taylor, C. W., A novel role for calmodulin: Ca^{2+}-independent inhibition of type-1 inositol trisphosphate receptors. *Biochem. J.*, 334, 447-455 (1998).

Choi, S., Rho, S. H., Jung, S. Y., Kim, S. C., Park, C. S., and Nah, S. Y., A novel activation of Ca^{2+}-activated Cl^- channel in *Xenopus* oocytes by ginseng saponins: evidence for the involvement of phospholipase C and intracellular Ca^{2+} mobilization. *Br. J. Pharmacol.*, 132, 641-648 (2001a).

Choi, S., Kim, H. J., Ko, Y. S., Jeong, S. W., Kim, Y. I., Simonds, W. F., Oh, J. W., and Nah, S. Y., $G\alpha_{q/11}$ coupled to mammalian phospholipase C b3-like enzyme mediates the ginsenoside effect on Ca^{2+}-activated Cl^- current in the *Xenopus* oocyte. *J. Biol. Chem.*, 276, 48797-48802 (2001b).

Dascal, N., Yekuel, R., and Oron, Y., Acetylcholine promotes progesterone-induced maturation of *Xenopus* oocytes. *J. Exp. Zool.*, 230, 131-135 (1984).

Fukunaga, K., Miyamoto, E., and Soderling T. R., Regulation of Ca^{2+}/calmodulin-dependent protein kinase II by brain gangliosides. *J. Neurochem.*, 54, 103-109 (1990).

Gnegy, M. E., Calmodulin in neurotransmitter and hormone action. *Ann. Rev. Pharmacol. Toxicol.*, 33, 45-70 (1993).

Hartzell, H. C., Activation of different Cl^- currents in *Xenopus* oocytes by Ca liberation from stores and by capacitative Ca Influx. *J. Gen. Physiol.*, 108, 157-175 (1996).

Jeong, S. M., Lee, J. H., Kim, S., Rhim, H., Lee B. H., Kim, J. H., Oh, J. W., and Lee, S. M., Ginseng saponins induce store-operated calcium entry in *Xenopus* oocytes. *Br. J. Pharmacol.*, 142, 585-593 (2004).

Kasri, N. N., Bultynck, G., Sienaert, I., Callewaert, G., Erneux, C., Missiaen, L., Pary, J. B., and De Smedt, H., The role of calmodulin for inositol 1,4,5-trisphosphate receptor function. *Biochim. Biophys. Acta*, 1600, 19-31 (2002).

Kuruma, A. and Hartzell, H. C., Dynamics of calcium regulation of chloride currents in *Xenopus* oocytes. *Am. J. Physiol.*, 276, C161-C175 (1999).

Lechleiter, J. D. and Clapham, D. E., Molecular mechanisms of intracellular calcium excitability in *Xenopus* laevis oocytes. *Cell*, 69, 283-294 (1992).

Lee, J. H., Jeong, S. M., Lee, B. H., Kim, D. H., Kim, J. H., Kim, J. I., and Nah, S. Y., Prevention of ginsenoside-induced desensitization of Ca^{2+}-activated Cl^- currents by microinjection of inositol hexakisphosphate ($InsP_6$) in *Xenopus laevis* oocytes: involvement of GRK2 and β-arrestin I. *J. Biol. Chem.*, 279, 9912-9921 (2004).

Liu, M., Chen, T. Y., Ahamed, B., Li, J., and Yau, K. W., Calcium-calmodulin modulation of the olfactory cyclic nucleotide-

gated cation channel. *Science,* 266, 1348-1354 (1994).

Matifat, F., Fournier, F., Lorca, T., Capony, J. P., Brule, G., and Collin, T., Involvement of the Ca^{2+}/calmodulin protein kinase II pathway in the Ca^{2+}-mediated regulation of the capacitative Ca^{2+} entry in *Xenopus* oocytes. *Biochem. J.,* 322, 267-272 (1997).

Missiaen, L., Parys, J. B., Weidema, A. F., Sipma, H., Valingen, S., De Smet, P., Callewaert, G., and De Smedt, H., The bell-shaped Ca^{2+} dependence of the inositol 1,4,5-trisphosphate-induced Ca^{2+} release is modulated by Ca^{2+}/calmodulin. *J. Biol. Chem.,* 274, 13748-13751 (1999).

Nah, S. Y., Ginseng, recent advances and trend. *Korea J. Ginseng Sci.,* 21, 1-12 (1997).

Parekh A. B., Interaction between capacitative Ca^{2+} influx and Ca^{2+}-dependent Cl^- currents in *Xenopus* oocytes. *Pflugers Arch-Eur. J. Physiol.,* 430, 954-963 (1995).

Parekh, A. B. and Penner, R., Store depletion and calcium influx. *Physiol. Rev.,* 77, 901-930 (1997).

Parys, J. B., Sernett, S. W., DeLisle, S., Snyder, P. M., Welsh, M. J., and Campbell, K. P., Isolation, characterization, and localization of the inositol 1,4,5-triphosphate receptor protein in Xenopus laevis oocytes. *J. Biol. Chem.,* 267, 18776-18782 (1992).

Sienaert, I., Kasri, N. N., Vanlingen, S. Parys, J. B., Callewaert, G., Messeian, L., and de Smedt, L., Localization and function of a calmodulin/apocalmodulin binding domain in the N-terminal part of the type-1 inositol 1,4,5-trisphosphate receptor. *Biochem. J.,* 365, 269-277 (2002).

Taylor, C. W. and Laude, A. J., IP_3 receptors and their regulation by calmodulin and cytosolic Ca^{2+}. *Cell Calcium,* 32, 321-334 (2002).

Yamada, M., Miyawaki, A., Saito, K., Nakajima, M., Yamamoto-Hino, Y., Ryo, T., Furuichi, T., and Mikoshiba, K., The calmodulin-binding domain in the mouse type 1 inositol 1,4,5-trisphosphate receptor. *Biochem. J.,* 308, 83-88 (1995).

Waxham, M. N. and Aronowski, J., Ca^{2+}/calmodulin-dependent protein kinase II is phosphorylated by protein kinase C *in vitro. Biochemistry,* 32, 2923-2930 (1993).